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Significance statement:

The statin drug lovastatin normalizes excessive protein synthesis and thereby ameliorates pathological changes in animal models of Fragile X Syndrome (FX), the most commonly identified genetic cause of autism. Recently, we compared the efficacy of lovastatin to the more potent and brain-penetrant drug simvastatin for correcting phenotypes in the *Fmr1*^{-y} mouse (Muscas et al., 2019). Surprisingly, we find simvastatin worsens excessive protein synthesis and has no impact on audiogenic seizures (AGS) in *Fmr1*^{-y} mice, suggesting it does not work in a similar fashion to lovastatin. A recent commentary by Ottenhoff et al. suggests that differences in dose and/or study design might account for our results. Here we discuss the points raised by Ottenhoff et al., as well as the evidence supporting a therapeutic role for lovastatin versus simvastatin. We conclude that differences between lovastatin and simvastatin warrant careful consideration with respect to the treatment of neurodevelopmental disorders.

Main text:

Therapeutic strategies that reduce protein synthesis have shown efficacy in reducing pathological brain phenotypes in FX (Stoppel et al., 2017; Protic et al., 2019). In the FX (*Fmr1*^{-y}) mouse model, lovastatin reduces the activation of Ras and downstream extracellular regulated-kinase (ERK) signalling, thereby normalizing protein synthesis and correcting changes in synaptic plasticity, neuronal hyperexcitability, epileptogenesis and learning (Osterweil et al., 2013; Sidorov et al., 2014) (**Table 1**). In the *Fmr1*^{-y} rat model, early administration of lovastatin prevents emergence of plasticity deficits and learning deficiencies later in development (Asiminas et al., 2019). In recent work we tested whether the structurally similar drug simvastatin could correct core phenotypes of excessive hippocampal protein synthesis and audiogenic seizures (AGS) in the *Fmr1*^{-y} mouse (Muscas et al., 2019). The motivation for testing simvastatin versus lovastatin is a two- to four-fold increase in potency, increased brain penetrance, and wider availability in Europe (Schachter, 2005). However, simvastatin has not been tested in any model of FX, and preclinical evidence of efficacy was required before incurring the significant cost of a clinical trial. This is particularly relevant for simvastatin, which has been tested for the treatment of Neurofibromatosis Type 1 (NF1), a neurodevelopmental disorder characterized by excessive Ras-ERK signalling. Early studies in the *Nf1*^{+/-} mouse showed a significant correction of several brain phenotypes with lovastatin (Li et al., 2005). Assuming the mechanisms for reversing pathological changes were identical for lovastatin and simvastatin, clinical trials were initiated for simvastatin in NF1 despite the absence of animal model studies. To date, three randomized placebo-controlled clinical trials for simvastatin in NF1

have failed to show a significant improvement in primary outcome measures (Krab et al., 2008; van der Vaart et al., 2013; Stivaros et al., 2018) (**Table 2**).

To our surprise, the comparison of lovastatin and simvastatin in the FX mouse model revealed significant differences. While lovastatin reduces protein synthesis in *Fmr1*^{-/-} hippocampus to WT levels, simvastatin resulted in a significant increase in protein synthesis in both genotypes (**Fig. 1A**). In contrast to lovastatin, simvastatin does not reduce ERK activation in *Fmr1*^{-/-} hippocampus, which is a key driver of the excess protein synthesis phenotype (Osterweil et al., 2010; Muscas et al., 2019). Moreover, simvastatin does not reduce the incidence of AGS in *Fmr1*^{-/-} mice, even when administered at a limiting high dose (**Fig. 1B**). In contrast, lovastatin treated cohorts show a significant reduction in seizure incidence, consistent with previous work (**Fig. 1C**) (Osterweil et al., 2013). From these results we conclude that lovastatin and simvastatin do not work in a similar fashion with respect to FX models, and suggest caution should be used when assuming these compounds are interchangeable. Our results have been discussed in a recent commentary by Ottenhoff et al., who have been involved in clinical trials with simvastatin for the treatment of NF1 (Krab et al., 2008; van der Vaart et al., 2013; Stivaros et al., 2018; Ottenhoff et al., 2020). The authors raise points regarding our study design, suggesting differences in dose and/or study design might account for the failure of simvastatin to correct *Fmr1*^{-/-} phenotypes. Here we discuss these points and examine the evidence supporting lovastatin versus simvastatin for the treatment of neurodevelopmental disorders.

Different actions on protein synthesis

Multiple treatments that normalize excess protein synthesis also ameliorate epileptogenic and behavioural phenotypes in FX models (Dolen et al., 2007; Liu et al., 2011; Gkogkas et al., 2014; Gantois et al., 2017; Stoppel et al., 2017). To investigate whether simvastatin corrects the excessive protein synthesis phenotype in the *Fmr1*^{-/-} mouse we utilized a metabolic labelling assay in hippocampal slices that has been employed in previous studies (Osterweil et al., 2010). As the potency of simvastatin is 2- to 4-fold that of lovastatin (Schaefer et al., 2004), we chose a starting dose of 5 μ M, which is half the 10 μ M starting dose of lovastatin used in previous work (Osterweil et al., 2013). Remarkably, this relatively modest dose of simvastatin caused a 50-60% increase in protein synthesis in both WT and *Fmr1*^{-/-} slices, dramatically worsening the protein synthesis phenotype (**Fig. 1A**) (Muscas et al., 2019). Given these results, we reasoned that increasing concentration would not only be ineffective, it would have deleterious consequences for both WT and *Fmr1*^{-/-} hippocampus. Instead, we tested whether a lower dose range of 0.1-0.5 μ M simvastatin might mitigate potential off-target effects and reduce the protein synthesis phenotype. Unfortunately, increased protein synthesis continued to be seen in slices treated at these lower doses

(**Fig. 1A**). In contrast, WT/*Fmr1*^{-/-} littermates treated with 50 μ M lovastatin resulted in the expected decrease in protein synthesis in *Fmr1*^{-/-} slices.

Looking at these results it is clear that under conditions where lovastatin normalizes protein synthesis in the *Fmr1*^{-/-} hippocampus, simvastatin causes a dramatic worsening of this core phenotype. Regarding these results, Ottenhoff et al. state:

“the most surprising finding of the study by Muscas and colleagues is the finding that simvastatin treatment at low dose actually worsened the Fmr1 phenotype by further increasing protein synthesis rates. (...) For the follow-up of these trials it would be of great importance to know if a comparable (low) dose of lovastatin (below the doses needed to inhibit ERK) would have a similar negative effect on this phenotype, especially since the dose that can be safely used in clinical trials is much lower than the in vivo dose used in this study”.

We note that dose-response studies have in fact shown that lovastatin decreases protein synthesis at 1, 10 and 20 μ M in cultured neuroblasts (Santa-Catalina et al., 2008). In hippocampal slices we have established that a lower dose of 10 μ M lovastatin does not cause a significant reduction in protein synthesis, however it certainly does not cause the dramatic increase seen with simvastatin (**Fig. 1A**) (Osterweil et al., 2013). In contrast, the impact of simvastatin on protein synthesis in neuronal cells has not been determined. The study cited by Ottenhoff et al. describes experiments performed in a muscle-derived C2C12 cell line, and it is not unreasonable to expect that the response in the nervous system will differ (Tuckow et al., 2011). Indeed, simvastatin has been shown to have a number of brain-specific effects that could contribute to the rise in protein synthesis, including a stimulation of neurotrophin release and augmentation of the expression and activation of NMDA-type glutamate receptors (NMDARs) (Parent et al., 2014; Roy et al., 2015; Chen et al., 2016). With respect to the latter, acute application of simvastatin has been shown to enhance surface expression and current flow through NMDARs in hippocampal slices, increasing the magnitude of long-term potentiation (LTP) (Parent et al., 2014; Chen et al., 2016). The changes in calcium influx and downstream signaling that are associated with NMDAR activation could contribute to the rise of protein synthesis we observe. In contrast, lovastatin has been shown to downregulate the GluN2B subunit of the NMDAR and thereby reduce associated signaling (Huo et al., 2014). This opposing action on NMDARs may contribute to the differential action on protein synthesis in hippocampal slices.

However, it should be noted that longer treatments with simvastatin, lovastatin and other statins reduce the production of cholesterol needed to stabilize NMDARs at the cell surface, ultimately causing a mild reduction in activity (Zacco et al., 2003; Ponce et al., 2008; Huo et al., 2014; McFarland et al., 2014). Therefore, longer-term experiments testing protein synthesis at multiple timepoints post simvastatin treatment are needed to determine whether changes in

NMDAR activity are involved. What we can conclude for now is that the differential impact of lovastatin and simvastatin on basal protein synthesis is striking, and should be investigated in follow up studies.

Different actions on ERK

Statins inhibit the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase pathway that produces both cholesterol and isoprenoid intermediates, which are important substrates for the posttranslational modification and activation of many proteins (Liao & Laufs 2005; Ling & Tejada-Simon 2016; Nürenberg & Volmer 2012). Lovastatin has been shown to inhibit the Ras farnesylation required for membrane association and subsequent activation of the ERK pathway (Schafer et al., 1989; Mendola and Backer, 1990; Li et al., 2005). In our comparison study we find that the low doses of simvastatin that raise protein synthesis have no significant impact on ERK activation in the *Fmr1*^{-/-} hippocampus (Muscas et al., 2019). Ottenhoff et al. argue that this result conflicts with previous work that shows “*like lovastatin, simvastatin has been shown to decrease ERK signalling* “. We note that the simvastatin dose used in our study is low due to the impact of higher doses on protein synthesis, and it may be that higher doses of simvastatin ultimately show an inhibitory effect on ERK. However, it is important to consider that the cited studies either do not measure ERK (Guillen et al., 2004; Ghittoni et al., 2006; Ghosh et al., 2009) , or show that simvastatin reduces ERK signalling in non-neuronal cells only when Ras-ERK is hyper-stimulated, but not under basal conditions (Furst et al., 2002; Miura et al., 2004; Ghittoni et al., 2005; Khanzada et al., 2006; Ogunwobi and Beales, 2008; Sundararaj et al., 2008; Kang et al., 2009; Chen et al., 2010; Lee et al., 2011; Takayama et al., 2011).

Unlike simvastatin, lovastatin has been shown to reduce basal Ras-ERK signalling in the absence of activation (Santa-Catalina et al., 2008; Osterweil et al., 2013). This point is particularly relevant to the protein synthesis phenotype in FX, which is not due to a hyperactivation of the ERK pathway but rather a hypersensitive response to normal levels of ERK signalling (Osterweil et al., 2010). It is also important to point out that clinical studies of platelets isolated from simvastatin-treated NF1 patients show no significant reduction in basal ERK activation (Stivaros et al., 2018), whereas those isolated from lovastatin-treated FX patients exhibit a robust reduction in ERK signalling that is correlated with treatment efficacy (Pellerin et al., 2016). Future studies examining the mechanistic differences between these statins could be particularly valuable for understanding the impact on neurological phenotypes.

Different actions on audiogenic seizures

The AGS phenotype has been used to test multiple potential pharmacological strategies that have moved on to clinical investigation in FX, including lovastatin (Yan et al., 2005; Liu et al., 2012; Busquets-Garcia et al., 2013; Osterweil et al., 2013; Gkogkas et al., 2014; Gantois et al., 2017; Stoppel et al., 2017). In Muscas et al. we compared acute injection of 100 mg/kg lovastatin to an equipotent dose of 50 mg/kg simvastatin. The results show a clear reduction in seizure incidence and severity with lovastatin, and no effect of simvastatin (**Fig. 1B**). Although Ottenhoff et al. argue “*there is no experiment in which lovastatin and simvastatin are compared at the same dose (and with the same vehicle)*”, the differential potency of these drugs has been well established (Schachter, 2005). If the question is whether there is an equivalent impact of these drugs, we would argue equivalent potency is a key point. Moreover, our attempts to increase simvastatin to 100 mg/kg revealed deleterious side effects that would have made it impossible to make a meaningful comparison.

Ottenhoff et al. bring up the important point that “*the dose in which a particular drug rescues a phenotype in animal model does not always translate into a clinically applicable and safe dose in humans.*” In our study we compared acute injections of relatively high doses of lovastatin and simvastatin due to the rapid action of these higher doses on the AGS phenotype (Osterweil et al., 2013). However, we also tested a lower dose of 3 mg/kg that is consistent with the dose given to humans according to standard calculations (Nair and Jacob, 2016)(**Fig. 1B**). Similar to the higher dose of simvastatin, the 3 mg/kg dose also failed to reduce seizures in the *Fmr1*^{-/-} mouse. In contrast, a range of lovastatin doses correct the AGS phenotype in *Fmr1*^{-/-} mice including a 2-day 10 mg/kg oral administration that is consistent with a human dose (**Fig. 1C**). This correction of AGS with lovastatin is seen whether *Fmr1*^{-/-} mice are bred on the FVB or C57BL6 background strains (Osterweil et al., 2013). Ottenhoff et al. argue “*if a behavioral rescue is observed in young mice (e.g. the rescue of seizures in Fmr1 mice was performed on P18-P29 mice, Osterweil et al., 2013; Muscas et al., 2019), it is important to investigate if such a rescue is still observed when the brain has fully matured.*” We note that multiple studies in mouse and rat models of FX and other neurodevelopmental disorders have shown that lovastatin corrects pathological phenotypes over a range of animal ages, including adults (**Table 1**). In contrast, beyond our study, there is no previous work examining simvastatin in any animal model of neurodevelopmental disorders including the *Nf1*^{+/-} mouse.

Study design

From the side-by-side experiments comparing lovastatin versus simvastatin we conclude there are differences in mechanism and efficacy that should be considered and further investigated in additional animal model studies. Ottenhoff et al. question whether the differences we report are in

fact significant, stating “the drugs should not only be tested side-by-side as interleaved experiments, they should also directly be compared with each other using a statistical analysis that tests for a main effect of treatment, and if significant, followed by a post-hoc analysis to compare the drugs”. Our experimental design compares lovastatin and simvastatin to matched vehicle groups, rather than directly to one another, because different concentrations of DMSO were needed for each drug. The blinded comparison of drug groups to counter-balanced vehicle controls is considered good practice by multiple authorities on experimental design for laboratory animals (Festing and Altman, 2002).

In order to evaluate the effects of lovastatin and simvastatin on seizure incidence we used a Fisher’s Exact test that allows for comparisons between small (< 50) nominal (yes/no) datasets, consistent with previous AGS studies (Pacey et al., 2009; Osterweil et al., 2010; Henderson et al., 2012; Michalon et al., 2012; Ronesi et al., 2012; Osterweil et al., 2013; Gross et al., 2015; Thomson et al., 2017). We find a significant difference in seizure incidence between vehicle and lovastatin-treated *Fmr1*^{-/-} mice (48%, $p = 0.0136$), but not vehicle vs low-dose simvastatin (0%, $p > 0.999$) or vehicle vs high-dose simvastatin (9%, $p = 0.6968$) (**Fig. 1C**). However, Ottenhoff et al. suggest that fitting our data to a logistical regression model is a better approach for determining global effects of treatment and genotype in all groups. They go on to fit our data to a model and state that it

*“shows a trend for a main effect of treatment ($\chi^2(6)=12$; $p=0.07$), but not for the interaction between genotype and treatment ($\chi^2(4)=4$; $p=0.3$). When performing a post-hoc Tukey’s test, neither the *Fmr1*-lovastatin versus *Fmr1* ‘low dose’ of simvastatin ($p= 0.96$) nor the *Fmr1*-lovastatin versus *Fmr1*-‘high dose’ of simvastatin treatment ($p>0.99$) are significantly different from each other. Hence, despite the fact that the lovastatin dose was 2-30 fold higher than simvastatin dose, it does not seem to perform significantly better than simvastatin in this seizure assay.”*

To investigate this issue, we examined the R script used to run the logistical regression model (shared by Ottenhoff et al.). Our analysis revealed a script error that led to the wrong reporting of p-values from the Tukey’s post-hoc tests. Running a corrected script shows lower p-values for the comparisons of lovastatin and simvastatin in *Fmr1*^{-/-} mice than originally published (**Table 3**). Additionally, Ottenhoff et al. run a Type 1 ANOVA that assumes an interaction between genotype and treatment, which we do not claim (nor can we with such a low incidence of seizures in WT). Re-running the logistical regression using a Type 2 ANOVA that does not assume an interaction shows a trend towards a main effect of treatment, though this does not reach significance ($p = 0.053$). However our original study was not powered to directly compare treatment groups, and we therefore investigated whether adding an additional treatment group would change the outcome of this analysis. In the original study testing lovastatin in *Fmr1*^{-/-} mice, multiple drug doses were tested

in both FVB and C57BL6 background strains (Osterweil et al., 2013) (**Fig. 1C**). After adding the data from the FVB group treated with 100 mg/kg lovastatin in this study, we re-ran the logistical regression and find a significant effect of treatment ($p = 0.00021$). When both lovastatin groups are collapsed, the significance of this effect increases ($p = 9.22 \times 10^{-5}$). Adding all lovastatin groups from Osterweil et al. 2013 increases the significance further ($p = 8.08 \times 10^{-9}$) (**Table 4**). Therefore, the logistical regression identifies the difference in treatment when given a dataset of sufficient size. Moreover, we find that a multinomial regression model that examines seizure severity scores reveals a significant treatment effect, even when applied to the original dataset from Muscas et al. 2019 ($p = 0.033$) (**Table 4; Extended data**). The important conclusion is that whether our results are analyzed directly or fit to a more complex model, they show that lovastatin corrects the AGS phenotype in *Fmr1*^{-y} mice, and simvastatin does not.

Future considerations

Our studies in *Fmr1*^{-y} animal models show promising results for lovastatin that are not seen with simvastatin. However, it is important to note that the role of statins in the treatment of Fragile X and other neurodevelopmental disorders will ultimately depend on large scale double-blind placebo-controlled trials. In the case of lovastatin, the results from double-blind placebo-controlled trials for NF1 are mixed, with one showing a significant improvement in verbal and nonverbal memory (Bearden et al., 2016), and another showing no significant effect on visuospatial learning and attention (Payne et al., 2016). In FX, a recent small-scale double-blind trial showed no additional effect of lovastatin on parent implemented language intervention (Thurman et al., 2020). For simvastatin, three randomized placebo controlled clinical trials have failed to show efficacy in NF1 (**Table 2**). At present, our study represents the only exploration of simvastatin in an animal model of neurodevelopmental disorders. We agree with Ottenhoff et al. that “*importance of looking at effective dosing ranges, and more detailed (in vivo) pharmacological studies in animal models should be performed to elucidate the dose-dependency of therapeutic benefit.*” Whether simvastatin shows benefits in FX or other models using a specific dosing regimen or alternative behavioral assays is an open question that would be very informative for future clinical studies. What is clear from our initial work is that there are significant differences between the action of lovastatin and simvastatin on brain function that warrant further attention.

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Figure and table legends:

Table 1. Animal model studies of lovastatin and simvastatin in neurodevelopmental disorders.

Studies using animal models of neurodevelopmental disorders have tested the impact of lovastatin on multiple phenotypes. Ours is the only study of simvastatin in a neurodevelopmental animal model.

Abbreviations: i.p.: intraperitoneal, s.c.: subcutaneous, mGluR-LTD: metabotropic glutamate receptor stimulated long-term depression, LTP: long-term potentiation, PFC: prefrontal cortex, ERK: extracellular-regulated kinase, MWM: Morris Water Maze, PPI: pre-pulse inhibition

Table 2. Human studies of lovastatin and simvastatin in neurodevelopmental disorders.

Lovastatin and simvastatin have been tested in clinical trials for FX and NF1, with varying outcomes.

Abbreviations: RCT: randomized placebo-controlled trial; ABC: aberrant behaviour checklist, CGI-S: clinical global impression scale, MRI: magnetic resonance imaging, ERK: extracellular-regulated kinase, GABA: gamma-Aminobutyric acid

Table 3. Reordered comparisons reveal correct p values for Tukey's post-hoc tests.

The regression model R script used by Ottenhoff et al. assigns different functions to set up the regression model matrix ("Unique") versus the Tukey's contrast matrix ("Tables"). This results in different order of groups for the two matrices which results in assignment of different headings to the test results. An altered version of the script with the factors level set in the same order for the model matrix and contrast matrix shows the correct Tukey's test results (see Extended Data). Estimate and z value are multiplied by -1 to reflect the corresponding tests headings. Reversed values are italicized and the corrected p-values reported by Ottenhoff are in bold.

Table 4. Regression model of AGS incidence and severity shows significant treatment effect in lovastatin versus simvastatin groups. Re-running the logistical regression comparing lovastatin and simvastatin treatments using a Type 2 ANVOA shows a non-significant trend towards an effect

of treatment. Adding data from the FVB 100 mg/kg lovastatin group originally published in Osterweil et al. 2013 shows a significant treatment effect either when kept separate or when collapsed into the existing lovastatin group. Adding data from additional lovastatin treatment groups from C57BL6 cohorts from Osterweil et al. 2013 (10 mg/kg, 30 mg/kg and 100 mg/kg) further increases the significance of the treatment effect. As the interaction of genotype and treatment does not reach significance using this model, it may be that lovastatin corrects seizures in both WT and *Fmr1*^{-/-} mice equally, however the low number of animals have seizures in the WT groups makes this difficult to assess. To compare lovastatin versus simvastatin treatment groups, a multinomial regression model of seizure severity scores with genotype and treatment effect was performed in R using the multinom function in the nnet package (see Extended Data).

Figure 1. Lovastatin, not simvastatin, corrects fragile X phenotypes. (A) Data from (Osterweil et al., 2013) and (Muscas et al., 2019) were combined and re-analysed. Metabolic labelling was performed on hippocampal slices prepared from WT/*Fmr1*^{-/-} littermates as previously described. A dose-response curve shows lovastatin corrects excess protein synthesis in the *Fmr1*^{-/-} hippocampus at 50 μ M (Two-way repeated measures mixed-model ANOVA treatment $p = 0.0052$, genotype $p = 0.0006$, genotype x treatment $p = 0.0438$; Sidak's WT veh vs KO veh $*p = 0.0021$, KO veh vs KO 50 $*p = 0.0014$). In contrast, simvastatin significantly raises protein synthesis in a dose-dependent manner in both *Fmr1*^{-/-} and WT hippocampus (Two-way repeated measures mixed-model ANOVA treatment $p < 0.0001$, genotype $p = 0.0005$, genotype x treatment $p = 0.9754$, Sidak's WT veh vs WT 0.5 $*p = 0.0120$, WT veh vs WT 5 $*p < 0.0001$, KO veh vs KO 0.5 $*p = 0.0157$, KO veh vs KO 5 $*p < 0.0001$). **(B)** Data re-plotted from (Muscas et al., 2019) (Figure 1-1). AGS assays show that acute injection of 100 mg/kg lovastatin significantly reduces the incidence of seizures in *Fmr1*^{-/-} mice versus vehicle control (Fisher's Exact test $*p = 0.0136$). Conversely, neither an equipotent dose of 50 mg/kg simvastatin (Fisher's Exact test $p = 0.6968$) nor a lower 3 mg/kg dose significantly (Fisher's Exact test $p > 0.999$) impacts the incidence of seizures in the *Fmr1*^{-/-} mouse. **(C)** AGS results from (Muscas et al., 2019) and (Osterweil et al., 2013) show that although simvastatin fails to reduce seizures, lovastatin significantly reduces seizures when given at 10 mg/kg orally (p.o.) for 2 days, 30 mg/kg injection i.p., or 100 mg/kg injection i.p. in *Fmr1*^{-/-} mice on both C57BL6 and FVB background strains (Fisher's Exact test: 10 mg/kg $*p = 0.003$, 30 mg/kg $*p = 0.041$, 100 mg/kg C57 $*p = 0.005$, 100 mg/kg FVB $*p = 0.005$) (Figure 1-2, Figure 1-3).

Extended Data:
R script for logistical regressions

Figure 1-1. Original dataset from Muscas et al. 2019

Figure 1-2. Combined dataset from Muscas et al. 2019 and 100 mg/kg dataset from Osterweil et al. 2013

Figure 1-3. Combined dataset from Muscas et al. 2019 and 4 datasets from Osterweil et al. 2013